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DISSERATION

The immunological Relationship and Inactivation of Various Penicillinases by Antipenicillinase Immune Serum

by

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TOPICAL OUTLINE OF THESIS

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INTRODUCTION

The purpose of the present problem is to determine the specificity of antipenicillinase immune serum. It has been shown that some organisms produce an enzyme, penicillinase, which is capable of inactivating penicillin. It has been demonstrated also that antipenicillinase immune serum can be produced. This report deals with the production of such an antiserum using penicillinase produced by one organism and the determination of its specificity for the penicillinases produced by three other organisms, by precipitin and inhibition tests.

REVIEW OF LITERATURE

Fleming[13] in 1929 observed that penicillin did not inhibit the growth of E. coli and a number of other bacteria belonging to the coli-typhoid group. Abraham and Chain[1] in 1940 investigated the cause of the resistance of these organisms to penicillin. An agent which was able to inactivate penicillin was extracted from crushed cells of E. coli. The activity of this agent was found to be destroyed by heating to 90 C for 5 minutes and by incubation with papain activated with potassium cyanide at pH 6. These investigators further found that the substance could be precipitated by alcohol and that it was nondialyzable through cellophane membranes. For these reasons they stated that this substance was an enzyme and named it penicillinase.

Penicillinase has been shown to be produced by Bacillus subtilis,[12,14,37,38] coliform bacilli,[5,26] Micrococcus lysodeikticus,[1,38] aërobic spore-forming bacilli,[5,14,38] paracolon organisms,[7,17,26,32,34] Escherichia coli,[1,7,17,20] Shigella dysenteriae,[7] Shigella paradysenteriae,[7]

Shigella sonnei,[7] Shigella sp. (newcastle),[7] Bacillus cereus,[3,7,26] Bacillus megatherium,[7,26] Bacillus anthracis,[7] Aërobacter aërogenes [7,17] Alcaligenes fecalis,[7] Pseudomonas (some strains),[7] Staphylococcus aureus (some strains),[6,23] and by Gram-negative rods found as contaminants of penicillin cultures.[1,28] Woodruff and Foster[38] found that cultures of B. mesentericus, Pasteurella sp., Mycobacterium tuberculosis, Actinomyces lavendulae, and Actinomyces antibioticus inhibited penicillin. These authors, who tested a large number of fungi and yeasts, reported that of the yeasts only two species, Mycoderma valida and Debaryomyces guilliermondii, and a single fungus, Papulaspora sp., were at all effective in destroying penicillin.

Benedict, Schmidt, and Coghill[3] tested 65 bacterial species for penicillinase production and found that the highest enzyme producer was an unclassified spore-former closely related to or identical with Bacillus cereus. These cultures were numbered NRRL B-568 and NRRL B-569. Bacillus cereus NRRL B-569 has been used extensively by other investigators [18,21,22,26,27,29] for the production of penicillinase, as the yields are much greater than those of any other organism which has been studied up to date.

Lawrence[24] stated that clarase and takadiastase were capable of inactivating penicillin, but after further work they found that the penicillin-destroying powers of these two enzymes were due to contamination by aërobic, spore-forming penicillinase-producing bacteria.[25] Stanley[36] found that only 1 of 5 lots of clarase possessed penicillin-destroying properties and concluded that these penicillin-destroying organisms were

chance, bacterial, air-borne contaminants. Kimes and White,[19] in a search for penicillin inhibitors, reported that the activity of penicillin was not appreciably affected by serum, blood, peptones, purulent material, carbohydrates, growth-factors, phospholipids, purines, amino acids, or tissue extracts, but found that filtrates of cultures of a number of bacterial species, which they did not name, did inactivate penicillin. It has been demonstrated that papain, lysozyme, urease, peptidases from porcine duodenal mucosa, polidase, emulsin, ficin, trypsin, pure carbonic anhydrase, crude alpha- and beta-amylase, and one lot of takadiastase were inactive as destroyers of penicillin, whereas concentrated pumpkin protease and high diastatic malt syrup showed some destructive activity.[3]

McQuarrie and Liebmann[28] defined a unit of penicillinase as that amount of enzyme, which in 11 ml of pH 7.0 solution containing 50 Oxford units of penicillin, will destroy in 1 hour at 37 C an amount of penicillin equal to 57.5 per cent of the penicillin recovered in the control. This unit has been used for the only commercial preparation available but is an awkward and unsatisfactory one. Other workers[3] have established the unit of penicillinase as the minimal amount of the enzyme which will destroy 50 per cent of 100 Oxford units of crystalline penicillin in 1.0 ml of pH 7.0 phosphate buffer in three hours at 30 C. Morgan and Campbell[29] in an effort to simplify the unit have defined a "dilution unit" as the dilution of enzyme in pH 7.0 phosphate buffer that causes 50 per cent inactivation of 1 unit of penicillin in 1 hour at 37 C as tested by a cup-plate assay. Other

units have been defined, since almost every investigator has set up his own standard. It is obvious that no definition of a unit has received wide acceptance and until a unit of penicillinase is defined and receives general recognition, potency determinations cannot be accurately compared. Furthermore, the expression of activity based on the inactivation of a number of units of penicillin by a given volume of penicillinase is meaningless, except when used for a given series of comparative tests that are carried out by the same technic.[34]

Perlstein and Liebmann[30,31] and later Housewright and Henry[22] produced an antipenicillinase immune serum by the repeated intravenous injection of penicillinase into rabbits and measured the antibody response by precipitin and inhibition tests. Both groups of workers demonstrated that this antipenicillinase immune serum was able to protect penicillin from destruction by the same penicillinase that was used as an antigen in the production of the immune serum. Normal rabbit serum did not possess this property. Housewright and Henry[22] stated that antipenicillinase immune serum produced by the injection of penicillinase elaborated by B. cereus B-569, inactivated penicillinase produced by B. cereus B-569 and by S. aureus Long III A. They concluded that penicillinases from these two sources were immunologically similar. This work was not carried any further.

EXPERIMENTAL WORK

PRODUCTION OF PENICILLINASE.- At present there is no standard technic for the production of penicillinase. The several methods described in the literature have advantages and disadvantages. The basic

problem is to secure an adequate yield of penicillinase as free as possible from foreign protein. The method used in the present paper and evolved after considerable trial and error has been found to be satisfactory.

In order to produce penicillinase relatively free of extraneous protein, a semisynthetic medium of low protein content was used. This medium had the following percentage composition: Casamino acids (Difco) 1.0, NaCl 2.0, glucose 0.6, K_2HPO_4 0.5, KH_2PO_4 0.4, $CaCl_2$ .2 H_2O 0.08, $FeSO_4$ .7 H_2O 0.001, $MgSO_4$ .7 H_2O 0.005, and $MnSO_4$. 4 H_2O 0.003.[21] The pH* was adjusted to 7 with NaOH and the medium autoclaved at 15 pounds pressure for 20 minutes.

Each organism used, with the exception of the acid-fast bacillus, was tested for growth in the casamino acid medium. A 16 hour broth culture of each was inoculated into the casamino acid medium and carried through two subsequent transplants in the same medium at 24 hour intervals. Growth was abundant for all organisms. Methylene blue and Gram stains were made at the time of each transplant to check the purity of the cultures.

Large 500 ml flasks containing 125 ml of medium were inoculated with 2 ml of a 16 hour broth culture of each organism. These cultures were checked by Gram and methylene blue stains. The acid-fast bacillus was the only exception. This organism had been growing for one week in tryptic digest broth with 1 per cent glycerol. A Ziehl-Neelsen stain showed the culture to be pure. Several pieces of the growth were

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* Beckman pH meter used throughout

placed directly on to bits of sterile cork floating in the flask. At the end of the incubational period another acid-fast stain was made to check the purity of the culture.

It has been shown that only minute quantities of penicillinase were produced in the absence of penicillin.[12,21,26] Housewright and Henry[21] obtained maximal yields of penicillinase from B. cereus in the casamino acid medium upon the addition of 200 units of penicillin per ml to the flask initially and again at 24 hours. In all the experiments here described, 200 units of penicillin sodium (Bristol) per 1 ml of medium were added 20 minutes after the medium was inoculated and an identical amount was added 24 hours later.

Consistently better yields of penicillinase were obtained when the pH of the medium was maintained between the limits of 6.5 and 8.5.[21, 26,38] In order to maintain the pH at these levels 1.0 g of $CaCO_3$ was added to each 500 ml flask of medium.[21] Repeated determinations on the various cultures showed that for each organism the pH remained within this range.

It had been determined that the peak yield of penicillinase elaborated by B. cereus occurs at 120 hours.[21] All flasks were accordingly incubated at 37 C for a period of 4 to 5 days and were shaken frequently during this time.

The cells and remaining $CaCO_3$ were separated from the medium by centrifugalization at 4,500 rpm at 50 F for 1 hour. The supernatant fluid was decanted and termed "Crude penicillinase". Cultures of this material were always negative. This material was used for

the qualitative test for penicillinase.

ISOLATION AND PURIFICATION OF THE ENZYME.- The problem of obtaining a purified penicillinase proved to be a very real one. Actually a protein is not admittedly pure until it has been crystallized. This was not undertaken nor has it ever been done with penicillinase. Some authors[3,21] admit that their penicillinase is impure. The method for the purification of Penicillinase Schenley is not reported in the literature. An unsuccessful attempt was made to learn this method through private communication.

Seitz filtration of the "Crude Penicillinase" was not attempted since it has been reported that penicillinase preparations lost most or all of their activity in passing through this type of filter.[3,21,26,28] McQuarrie and Liebmann[28] proved that penicillinase was adsorbed on the asbestos filter pad. Dialysis was tried and resulted in a considerable loss of activity as has been the experience of others.[3,12,21,28,38] LePage and his co-workers,[26] on the other hand, were able to carry out dialysis without loss of penicillinase activity. Numerous methods for the adsorption and elution of penicillinase have been reported.[21,28,38] Activated alumina (mesh minus 80) was tried as an adsorbent, since McQuarrie and Liebmann[28] had reported that it was a fair adsorbent at pH 7. Housewirght and Henry[21] demonstrated adsorption onto alumina (90 mesh, 5 per cent suspension, pH 5.2) but could not elute any penicillinase with 2 per cent NaCl at pH 6.0. Alumina was tried since it was felt that the penicillinase might elute at pH 8.5. The alumina was placed in an oven at 400 F for 4 hours to dry. "Crude Penicillinase" of B. cereus was filtered

through a fritted glass filter. Then 10 g of alumina were added to 50 ml of the fluid. The preparation was stirred for 1 hour in an ice bath and centrifuged for 10 minutes. Assays showed that penicillinase was still present in the supernate. The alumina was washed twice in sterile distilled water. The alumina was eluted twice with phosphate buffer, pH 8.5, 40 ml being used for the first elution and 20 ml for the second. The adsorbent was separated each time from the buffer by centrifuging. Penicillinase was recovered in both eluates but there was considerable loss of the enzyme. Adsorption onto Hyflo- Super-Cel was tried as described by LePage, Morgan and Campbell[26] and was reasonably satisfactory. However, the method was cumbersome. There are several methods reported for isolation of the enzyme by protein precipitants which have met with varying success.[17,23,28,38]

Morgan and Campbell[29] reported a method of isolating the penicillinase by acid precipitation with subsequent filtration through a fritted glass filter. This method was adopted and was found to be satisfactory for the present work. The "Crude Penicillinase" was prepared by growing the culture in casamino acid medium with the addition of penicillin and $CaCO_3$. The bacterial cells and $CaCO_3$ were removed by high speed centrifugalization in the cold. The "Crude Penicillinase" was adjusted to pH 5.2 with glacial acetic acid. In each case the precipitated enzyme produced a very fine diffuse turbidity. Filtration of 100 ml was carried out immediately on a fritted glass filter of F porosity. The precipitated enzyme held on the surface of the filter was redissolved

in 10 ml of 0.5 per cent ammonia water and allowed to pass through the filter. By this method the penicillinase was concentrated 10 times. The pH was adjusted to 7.0 with NaOH and the final product was stored at 5 C. The penicillinases made were all stable over a period of four months as shown by repeated assays.

METHODS OF ASSAY OF PENICILLIN AND PENICILLINASE.- The method used in the assays below was based on the principle originally described by the Oxford group.[2] It consisted of the addition of penicillin in glass cylinders to growing broth cultures of Staphylococcus aureus which previously had been layered over an agar plate. The diameter of the zone of inhibition was used as the index of concentration of the penicillin and was compared to a known standard. Substantial modification in the procedure has since been made. The method of Foster and Woodruff[15] for the cup assay for penicillin was followed for the most part in this work for assaying residual penicillin in mixtures of penicillinase and penicillin. The cup assay was chosen rather than a tube dilution method[33] because it produced no false positive, was easier to handle, offered less chance for contamination, and a more accurate specific value was always obtained instead of the range characteristic of dilution methods.

Throughout the entire procedure great effort was made to thoroughly standardize the procedure and to minimize the variations caused by changes in medium, amount and condition of inoculum, incubation time, temperature, and other possible factors. A penicillin assay was placed on each plate, which in every case agreed with the standard. Penicillin solution for the day's assays were made up from a previously unopened

vial[26] with sterile distilled water and kept cold at all times. It was assumed that standards and unknowns would both be equally affected by any slight deviation. The results within this series of assays must be compared within themselves to show that the activity of a sample is due to the presence or absence of penicillinase as indicated by the zone of inhibition. Aseptic measures were strictly adhered to and all equipment was thoroughly cleaned and sterilized before use. All glassware and procelain equipment was heated to 80 to 90 C in concentrated H_2SO_4, to which a few crystals of KNO_3 were added daily. It was then washed thoroughly in warm soapy water, rinsed three times in distilled water, and sterilized with dry heat. Equipment was kept sterile until used. Hot H_2SO_4 and KNO_3 was forced through the fritted glass filters, and after a thorough rinse in water, a quantity of distilled water was run through until the pH of the filtrate was that of the distilled water. Ordinary cleaning solution, $H_2SO_4 \cdot K_2Cr_2O_7$, cannot be used, since slight traces of residual chromium ion may partially inactivate low concentrations of penicillin.[15]

Test Organism and Preparation of the Spore Inoculum.- The test organism for all the assays was a strain of B. subtilis obtained from Foster and Woodruff.[15] A spore suspension of this organism was prepared as by these investigators.[15] Then 150 ml of standard nutrient broth* in a 250 ml flask were inoculated from the slant culture of B. subtilis and incubated at room temperature with occasional shaking. An abundance

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* Peptone (Difco) 1 per cent; Beef extract (Difco), 0.3 per cent; NaCl, 0.5 per cent; Distilled water

of spores was observed by microscopical examination on the eight day and the culture was heated at 60 C for 30 minutes to destroy any viable vegetative cells. Various concentrations of the spore suspension were tested on agar plates to determine the optimal inoculum. The addition of 0.1 ml of spore suspension to 100 ml of agar gave a bacterial density in which the individual minute colonies were not confluent with one another. The zones of inhibition had sharp edges and the opaque plate provided good contrast. This spore suspension was stored in the cold and used for all assays, thus eliminating variation in the amount and condition of the inoculum.

Preparation of the Plates.-The Petri dishes which were used were somewhat deeper than the usual ones. The pyrex bottom was 100 x 20 mm. Unglazed porcelain covers were used, which enabled the water of condensation to escape during incubation and not drop back onto the agar. Dehydrated nutrient agar Bacto (for milk analysis) had been prepared with distilled water in 300 ml lots and autoclaved. The 300 ml of cooled agar (50 to 60 C) were seeded with 0.3 ml of the spore suspension and shaken thoroughly. By means of wide-mouth pipettes 22 ml of the seeded agar were transferred to each plate. The plates were rotated gently on a level surface to distribute the agar evenly and the agar was allowed to harden. They were placed in the refrigerator immediately, where they remained for a period not exceeding 3 hours. No more than 12 plates were made up at a time.

Setting up the Cups.- 20 minutes before the material to be assayed was ready the plates were removed from the refrigerator and small sterile porcelain cups, previously inspected for cracks or chips, were planted on the agar. The outer diameter of these cups measured 8 mm and the internal diameter 6 mm. Forceps were flamed and the cups were picked up individually. One end was held for 7 seconds in the flame and placed lightly on the agar. Six cups were arranged in a circle equidistantly from the center on each plate. During this performance each dish was placed on a drawing of the desired arrangement. The heated cup melted the agar which hardened immediately and formed a seal. If the cup had been jarred or leaked, it was detected at the time of reading by an abnormally large or imperfect circular zone of inhibition. The control cup was designated by a check mark with a wax pencil on the under side of the dish. Cups were filled in counterclockwise rotation from this cup beginning with the highest dilution.

Filling the Cups.- The cups were filled with the material to be assayed by means of sterile medicine droppers of uniform size and bore. When several dilutions were assayed, a single dropper was used for each specimen. The first cup to be filled was the one containing the highest dilution. The sterile dropper was rinsed three times with the solution and the first cups on each of duplicate plates were filled to the top. The dropper was then rinsed three times in sterile distilled water and three times with the next lower dilution and the second cups on the duplicate plates were filled with the same number of drops. This process was repeated until all the cups were filled with the various dilutions. The

penicillin control was put on all plates at the same time. The plates were placed in the incubator immediately. The filling time never exceeded one half an hour. All assays were incubated at 30 degrees C for 20 hours, at which time the zones of inhibition were measured.

Measurement of the Zones of Inhibition.- A colony counter was used for the viewing box. This provided a black background with indirect light so that the zones stood out clearly. A clamp held the plate in place and a magnifying glass was above the plate. A millimeter rule was slipped under the plate and the zone was measured and recorded. Duplicate plates were made for each assay. The results were compared and averaged. The corresponding zones of inhibition on each plate were remarkably consistent. If a serious discrepancy occurred, the assay was repeated.

Qualitative Assay for Penicillinase.- The following method was used to determine the presence or absence of penicillinase in the material termed "Crude Penicillinase". To 4.2 ml volumes of the unknown was added 0.3 ml of a dilution of penicillin (1.5 units). The control solution contained 4.2 ml of casamino acid medium was 0.3 ml penicillin dilution (1.5 units). These tubes were incubated at 37 C for one hour and the contents put on the seeded agar plates. The period of incubation was 20 hours at 30 C. The zones of inhibition of the mixtures tested were measured and compared with the zone of inhibition of the control penicillin cup. When the zone of inhibition was reduced it was considered evidence of the presence of penicillinase. If penicillinase was not detected in appreciable quantities by this assay, the organisms were discarded.

Quantitative Assay of Penicillinase.- A quantitative method for the assay of penicillinase was set up in the following manner. A 1 ml sample of "Purified Penicillinase" was pipetted into a 20 x 100 mm test tube and 8 ml of 1 per cent pH 7 phosphate buffer were added. From this tube, dilutions of 1:10, 1:20, 1:40, 1:80 and 1:100 were made by diluting with the buffer. Each tube then contained 4.5 ml. A control tube containing 4.5 ml of the buffer was added to the series. To each tube 0.5 ml of buffer was added. In the assays with antibody-antigen complex, to be described later, it was here that 0.5 ml of the anti-penicillinase immune serum was added instead of the buffer and the tubes were incubated for 1 hour at 37 C. To each tube 0.5 ml of penicillin dilution or 25 units were added. The tubes were incubated at 37 C for 1 hour. The mixtures were then plated as described above. Various dilutions were tried until a range was found that was satisfactors for all of the chosen penicillinases. This method was similar to that used by McQuarrie and his associates.[28]

For the purpose of assay, the B. cereus Penicillinase Schenley (see p.16) was diluted with sterile water so that 1 ml contained 50 units (McQuarrie and Liebmann unit[28]). This dilution fell into the range used for the quantitative assays of the other four penicillinases and had the advantage of affording an index for comparison of the strengths of the other penicillinases.

Preparation of 1 per cent pH 7 Phosphate Buffer.- The 1 per cent pH 7.0 phosphate buffer was prepared as follows: 0.363g KH_2PO_4 and 0.713g

$Na_2HPO_4 \cdot 2 H_2O$ were dissolved in sterile distilled water and the final volume brought to 100 ml. The pH was 7. If adjustment had to be made, it was done by adding a minute amount of one salt or the other.

Standardization of Penicillin.- The penicillin used was standardized on two successive days. Two previously unopened vials, designated A and B, were used. A series of 6 (20 x 100 mm) test tubes was set up. Dilutions of penicillin were made with sterile distilled water so that the desired number of units contained in 0.5 ml of water was present in the proper tube. To each tube 5 ml of the phosphate buffer were added. The tubes were incubated for 1 hour at 37 C and the solutions dropped into cups on seeded plates. A separate sterile dropper was used for each tube in this case. The results were identical (Table I). A standard curve was constructed by plotting the diameters of the zones of inhibition on the ordinate and the number of units of penicillin on the abscissa (Fig. 2). It should be noted that each penicillinase assay was controlled by an assay of penicillin and in each instance the control penicillin assay agreed with this standard.

ORGANISMS TESTED FOR THEIR PRODUCTION OF PENICILLINASE.- Thirteen organisms were studied to determine their ability to produce penicillinase. These organisms were Bacillus cereus NRRL B-569,[a] a rapidly growing, non-pathogenic, acid-fast bacillus,[b] Bacillus megatherium,[c] Aërobacter aërogenes,[c] Escherichia coli communior,[d] Escherichia coli communis,[e] Bacillus anthracis,[e] Shigella dysenteriae,[e] Shigella paradysenteriae,[e] Paracolon intermediate,[e] Bacillus subtilis,[e] Bacillus subtilis (Marburg strain),[e] and Staphylococcus aureus.[3e]

a.Obtained from Dr.R.D.Coghill of the Northern Regional Research Laboratories
b.Obtained from Dr. Alice Marston, Boston University School of Medicine
c.Obtained from Catherine C. Dietz, Temple University School of Medicine
d.Obtained from Dr. Manson Meads, Harvard Medical School
e.Obtained from Harvard Medical School

Qualitative assays were done on the culture fluid called "Crude Penicillinase" of each organism. S. aureus and Shigella para-dysenteriae did not produce any penicillinase detectable by this method (Table II) and were discarded. Both strains of B. subtilis, Shigella dysenteriae, and B. anthracis produced only very small amounts of the enzyme. Because of this it was not feasible to include them in the study and they, too, were discarded. The "Crude Penicillinase" of each of the 7 remaining organisms was quantitatively assayed. Four organisms were found to produce penicillinase in quantities sufficient to permit further study. These were B. cereus, the acid-fast bacillus, B. megatherium, and Aërob. aërogenes.

PRODUCTION OF ANTIPENICILLINASE IMMUNE SERUM.- Eight adult male rabbits were injected intravenously with penicillinase produced by B. cereus NRRL B-569. This penicillinase was obtained from the Schenley Laboratories. The quantities of purified penicillinase required for the immunization of rabbits were so large that the production of penicillinase for this purpose in this laboratory was impractical both from a mechanical and an economical point of view. The equipment for handling and producing the large quantities of medium necessary was unavailable and the cost of the materials was prohibitive. B. cereus is by far the best penicillinase producer and its penicillinase is the only one which has been produced commercially. The B. cereus Penicillinase Schenley was obtained in dried form in sterile vials containing 1000 units (McQuarrie and Liebmann unit[28]). All penicillinase used was of the same lot (Control No. 11S200). Each 1000 units was diluted with either 20 ml of sterile distilled water or 20 ml of sterile physiological saline solution. Assays showed that the

strength was the same with both diluting fluids.

The plan of immunization used by Housewright and Henry[21] was the first to be tried (Table III). Rabbits #1 and #2 were given initial doses of 360 units (McQuarrie and Liebmann unit[28]). Rabbits #3 and #4 were given initial doses of 720 units in an effort to shorten the immunization period. Three rabbits died within 24 hours and the other rabbit, receiving the larger dose within 96 hours. Presumably these rabbits died either because of toxins commonly produced by Gram-negative organisms which may have been present as contaminants in the penicillinase or possibly because the penicillinase itself was toxic. A third possible cause of death would be intravascular hemolysis due to the distilled water used to dilute the penicillinase. The considerable weight loss and rather poor condition of the rabbits which survived carefully graded doses of penicillinase indicates the toxic nature of the material. Since the method of immunization advocated by Perlstein and Liebmann[31] required still larger doses, it was not tried.

Immunization was attempted successfully by the intravenous injection of small daily and bi-daily doses, which were gradually increased in size as the condition of the animals warranted (Table IV). Two albino rabbits, #5 and #6, were given daily doses of 100 penicillinase units (McQuarrie and Liebmann unit[28]). These doses were gradually increased until each rabbit was receiving 200 units daily. The rabbits were carried at this level with occasional rest periods and trial bleedings until rabbit #5 had received 9950 units and rabbit #6 4200 units. Rabbits #7 and #8 were given 50 units twice daily. The dose was gradu-

ally increased until they were receiving 125 units in the morning and 100 units in the evening. The rabbits received a total dose of 9925 and 9825 units respectively.

Precipitin tests and cup assay inhibition tests on blood from trial bleedings indicated that maximal antibody titers occurred at 4 weeks (a three week course of injections and a one week rest period) (Table IV, rabbit #6), and after courses of injections followed by booster doses given over a period of 5 days followed by a two-week rest period (Table IV, rabbits #5,#6,#7, and #8). Blood was obtained in the trial bleedings by allowing approximately 20 ml of blood to drip from the marginal ear vein directly into a 50 ml sterile pointed centrifuge tube. The ear was prepared by shaving the fur, washing the skin with soap and water, and applying alcohol. The vein was cut crosswise. Care was taken to avoid contamination. Xylene was applied above the cut to hasten the flow. The rabbits were fasted before all bleedings to avoid, as far as possible, the presence of fat or opalescence in the serum which would interfere with the readings of the precipitin tests. In the trial bleedings of Nov. 2nd, Nov. 8th, and Nov. 20th all titers were high. Booster doses were indicated on Dec. 9th because of the unavoidably long rest periods of each animal. Assays on the trial bleedings of Dec. 22nd showed a high titer. The rabbits were given a two week rest period following the last booster injection. One rabbit, #7, was found dead during the rest period. Approximately 100 ml of blood was withdrawn by cardiac puncture from each of the remaining three animals. The blood was kept at room temperature for 6 hours to allow the clots to retract and was placed in the refrigerator over night. The following morning the clots

were ringed, and the sera cleared by centrifugalization for one half an hour and pipetted off. Each serum was allowed to "age" one week. Inhibition tests and precipitation tests were done on the separated sera.

Normal sera, which were used as controls, were obtained from six albino rabbits of mixed sex with weights of from 3 to 5 kilograms. These were designated as N_1 - N_6. They were fasted and bled through the marginal ear vein with the exception of N_3 and N_4 in which cases the blood was obtained directly from the heart. The sera were prepared by the method described above.

TESTS TO DETERMINE THE EFFECT OF ANTIPENICILLINASE IMMUNE SERUM UPON PENICILLINASE.- Two sets of experiments were carried out in an effort to determine the effect of antipenicillinase immune serum upon the various penicillinases. These experiments took the form of precipitin tests and inhibition tests.

Precipitin Test.- Perlstein and Liebmann[31] reported the successful use of a precipitin test for the testing of antipenicillinase immune serum and purified penicillinase. Their method was the serum dilution one of Culbertson.[11] Housewright and Henry[22] also made use of a precipitin test.

A capillary-tube "ring" test was used for these experiments. This was a modification of the "ring" test for the precipitin reaction.[9] The reagents were antipenicillinase immune serum or normal serum and penicillinase. The antipenicillinase immune serum and the normal serum

were used undiluted and in two-fold dilutions up to 1:64. Sterile physiological saline solution was used to make the dilutions. Successive dilutions of the penicillinases were made in the same way except that the penicillinases were not used undiluted. Dilutions were made in 13 x 100 mm test tubes using serological pipettes. A saline control was added to each series. The 3 inch capillary tubes were then filled by touching the tube to the surface of the desired dilution of the antibody allowing the fluid to rise half way up in the bore and then touching the tube to the surfaces of the desired antigen dilution allowing the tube to fill within 2 mm of the top. An interface free from air bubbles was achieved. The capillary tube was supported by placing the lower end into plasticene in a small shallow container. The tubes were incubated at 37 C for 20 minutes and read against a black background with an indirect light. A positive reaction showed a definite white zone or plane at the junction of the two clear fluids. The tubes were allowed to stand overnight at 37 C and reread. The zone at this time was more definite though it was very unusual for a tube to be positive at this time when it had been negative at the 20-minute reading.

Inhibition Test.- In order to determine the effect of antipenicillinase immune serum on penicillinase, these two substances were allowed to act upon each other. The mixture was then assayed with penicillin to determine the amount of penicillinase remaining. The test used was a modification of tests described by Perlstein and Liebmann[30] and by Housewright and Henry.[22] The tests were set up as follows. The penicillinase was diluted as described above under Quantitative Assay of Penicillinase.

Each tube contained 4.5 ml of the penicillinase dilution. A control tube containing 4.5 ml of the buffer was added to the series. Then 0.5 ml of the antipenicillinase immune serum was added to each tube. The tubes were incubated for 1 hour at 37 C. In the control tests, described as "normal serum" and "no serum", 0.5 ml of normal serum was added in the former and 0.5 ml of the phosphate buffer was added in the latter instead of the antipenicillinase immune serum. All tubes were incubated at 37 C for 1 hour. Then to each tube was added 0.5 ml of penicillin dilution or 25 units. The tubes were reincubated at 37 C for 1 hour. The mixtures were then assayed to determine the amount of penicillin destroyed by the remaining penicillinase. The various normal sera were used in rotation for these assays since there was no significant difference in their reactions in trial assays when each normal serum was tested against antipenicillinase immune serum and the same penicillinase.

EXPERIMENTAL RESULTS

Precipitin tests were done using the penicillinases of B. cereus (Schenley), B. cereus, B. megatherium, the acid-fast bacillus, and Aërob. aerogenes and the antipenicillinase immune serum of rabbit #6 (Tables V - IX). These were controlled with normal rabbit serum. The results were confirmed in all respects when spot assays were carried out using antipenicillinase immune sera from rabbits #5 and #8.

Examination of the tables shows that precipitation occurred in high dilutions of the antipenicillinase immune sera and penicillinases

of B. cereus (Schenley), B. cereus, and B. megatherium. This was in marked contrast to the results when the penicillinases of the acid-fast bacillus and Aërob. aerogenes were used. In these last two there was no significant difference between the antipenicillinase immune serum, the normal serum, and the phosphate buffer. The positive results in the control of all tests when the normal sera were undiluted may be due to the presence of fat in the sera or it may represent a physical-chemical phenomenon. In any event it does not affect the validity of the results.

Inhibition tests were carried out on five penicillinases produced by the following organisms: B. cereus (Schenley), B. cereus, B. megatherium, the acid-fast bacillus, and Aërob. aërogenes (Tables X - XIV). In each case the quantity of antipenicillinase immune serum was constant and the penicillinase was diluted. The test was controlled in two ways: (1) normal serum was substituted for the antipenicillinase immune serum; and (2) phosphate buffer was substituted for the anti-penicillinase immune serum.

B. cereus penicillinase (Schenley) was tested against antipenicillinase immune sera #5, #6, #7, and #8. The other penicillinases were tested against sera #5, #6, and #8. Only a small amount of serum was taken from rabbit #7 during a trial bleeding and the rabbit died before a larger amount could be obtained.

The assays reported for the penicillinase of each organism against the antipenicillinase immune sera were done on one day in order to keep the conditions of the test standard. For example in Table XII, one series of tubes was run for "Normal Serum" and one series of tubes

for "No Serum". A series for each of the antipenicillinase immune sera was run. Results were verified by repeating the entire procedure at another time when essentially the same results were obtained.

It can be seen (Tables X to XIV) that the B. cereus antipenicillinase immune sera inactivated the penicillinases of B. cereus (Schenley), B. cereus, and B. megatherium. In each instance the penicillinase was inactivated and the penicillin was free to inhibit the growth of the test organism. This is in marked contrast to the results obtained when normal sera and phosphate buffer were substituted for the antipenicillinase immune sera. In these tables there is a slight variation between the readings of "Normal Serum" and those of "No Serum". This finding was not a consistent one throughout the assays since it was not found in those on the penicillinases of the acid-fast bacillus and Aërob. aërogenes. This may be due to experimental error or it may be that normal serum per se enhances the effect of these penicillinases.

Antipenicillinase immune sera did not react with the penicillinases of the acid-fast bacillus or Aërob. aërogenes. These assays were clear cut and there was no variation between the assays using antipenicillinase immune serum and control assays. The results of the inhibition tests were in complete agreement with those of the precipitin tests.

DISCUSSION

Penicillinase is an enzyme which has the property of inactivating penicillin. It may be either extracellular or intracellular and when both forms are produced by one organism they are identical as far as is known. Henry and Housewright[18] showed that penicillinase is a protein or has a protein component essential for activity although it

is not a basic protein. They conclude that penicillinase is not a basic protein from the fact that desoxyribonucleic acid but not ribonucleic acid is capable of forming relatively stable salts with basic proteins and no inhibition of penicillinase activity was observed with either compound. However, as they point out, the possibility remains that such a complex may retain enzymic activity. Penicillinase may be considered as a constitutive enzyme as it may be produced by bacteria in the presence or absence of penicillin.[38]

There is a wide variation in the values given in the literature for the optimal temperature and pH range for the activity of penicillinase. It is likewise true for some of the chemical properties of penicillinase, where even conflicting and contradictory results are reported. In all probability this is due to the fact that almost every investigator used a different organism and a different method for the production of penicillinase. Generally speaking, the optimal pH for enzymic activity is considered to be 7.0 with the narrow range of 6.8 to 8, the range at which penicillin is reported to be stable. The activity is destroyed at pH 2.[38] The optimal temperature is 37 C,[38] with the range 29 to 37 C. The rate of inactivation is retarded but positive at 0 C.[38] The enzyme is thermolabile. With a single exception,[12] authors agree that the activity is destroyed by heating at 80 C for 30 to 60 minutes.[32,34,37] Activity is reduced or destroyed by heating to any temperature over 45 C.[3,18,28,38]

Penicillinase is stable in sealed vials in the dried state.[34]

Wet preparations show various degrees of stability at various pH levels and at low temperatures over periods of 4 to 13 weeks.[21,29,32,34,37,38]

Penicillinase from B. cereus will inactivate penicillins G and X with equal facility and penicillins F and K at a lower rate.[18] Oxygen is not taken up during the reaction between penicillin and penicillinase, and penicillinase is able to inactivate penicillin equally well under aërobic and anaërobic conditions.[1,37] It has been shown further that when penicillin is acted upon by penicillinase in the presence of bicarbonate, CO_2 is given off suggesting that a new acidic group is liberated.[14] Proom[32] added a minute quantity of penicillinase to penicillin in solution and found the latter to be inactivated. Five days later he added an equal amount of penicillin to this same mixture which was also inactivated. From this experiment he concluded that penicillinase acts as a catalyst.

Penicillinase shows a high degree of specificity for the configuration of the basic penicillin molecule. This was demonstrated by Henry and Housewright[18] who set up a manometric method for the assay of penicillinase based on the formation in the penicillin molecule of a carboxyl group from the ring carbonyl group that is adjacent to a ring nitrogen. This same configuration also occurs in certain purines, pyrimidines, and other naturally occurring substances. When penicillinase was assayed against xanthine, adenine sulfate, guanine, riboflavin, sodium pyruvate, and uracil, there was no evidence of a significant reaction.

Considerable attention has been directed to the possibility that production of penicillinase by an organism may be the determining factor in that organism's resistance or sensitivity to penicillin.

Kirby[23] found that seven "naturally" penicillin-resistant strains of S. aureus had penicillinase activity while no such activity was found in seven penicillin-sensitive strains of this organism. Bondi and Dietz,[6] in a study of 115 strains of staphylococci, showed that 13.9 per cent were penicillin-resistant and produced penicillinase whereas all the susceptible strains failed to produce the enzyme. It has been shown, however, that most of the primary pathogenic organisms do not produce penicillinase regardless of their sensitivity or insensitivity to penicillin.[8] Furthermore, two organisms have been found which produce penicillinase and are also slightly susceptible to penicillin.[8]

In attempts to clarify this question several studies have been carried out to determine if bacteria made resistant to penicillin developed the ability to produce penicillinase. Spink and Ferris[35] found that strains of staphylococci which became penicillin-resistant in the human body produced penicillinase but strains of staphylococci made resistant in vitro did not develop the ability to make the enzyme. Abraham et al[2] were unable also to demonstrate penicillinase in staphylococci made penicillin-resistant in vitro. Bondi and Dietz[8] showed that none of their cultures of S. aureus, E. typhosa, and P. vulgaris developed the ability to make penicillinase when made penicillin-resistant in vitro. In addition, they showed that even high concentrations of penicillin did not inhibit the growth of certain penicillinase-negative organisms such as species of Pseudomonas and B. melitensis. It would appear then that organisms made resistant to penicillin in vitro do not develop the ability to make penicillinase. Further, the ability to make penicillinase is

probably either a natural one inherent in the particular organism or, if the work of Spink and Ferris[35] is confirmed, this ability is one that may be acquired *in vivo*. From the evidence at hand it would seem that an organism's ability to resist penicillin is not dependent upon the production of penicillinase by the organism although, when penicillinase is present, this is an important factor in the organism's ability to resist penicillin.

The antipenicillinase immune serum produced against *B. cereus* inactivated the penicillinases produced by *B. cereus* and *B. megatherium*. There was no evidence that the antipenicillinase immune serum of *B. cereus* inactivated the penicillinases produced by *Aërob. aërogenes* and the acid-fast bacillus. These findings were confirmed by the precipitin tests in which *B. cereus* antipenicillinase immune serum reacted strongly with *B. cereus* and *B. megatherium* penicillinases while no significant reactions took place with the penicillinases of *Aërob. aërogenes* and the acid-fast bacillus. From these results it can be concluded that penicillinases of *B. cereus* and *B. megatherium* are immunologically similar and the penicillinases of *Aërob. aërogenes* and the acid-fast bacillus are immunologically dissimilar to the penicillinase of *B. cereus*.

It is not surprising to find that the penicillinases of *B. cereus* and *B. megatherium* are immunologically similar since these two organisms are closely related both belonging to the family *Bacillaceae* and the genus *Bacillus*.[4] Further, the organisms are so closely related that intermediate forms between the two have been described. The other two organisms tested have little in common with *B. cereus*.

It is interesting to compare these findings to those of Housewright and Henry[32] who state that the B. cereus antipenicillinase immune serum inactivated penicillinases produced by B. cereus and the completely unrelated organism S. aureus Long III A. This statement* is not documented and there is no way to judge the degree of inhibition.

Antipenicillinase immune serum contains a specific antibody, antipenicillinase, which has the property of inhibiting the action of the enzyme penicillinase. Two possible mechanisms for this ability to inactivate penicillinase can be postulated. One, suggested by Perlstein and Liebmann,[30] is that penicillin combines with the plasma proteins of the antipenicillinase immune serum to form a penicillin protein complex capable of inactivating penicillinase. Against this theory, there is the finding by Chow and McKee[10] that penicillin combines with normal albumin but not normal globulin. Since all antibodies studied to date have been shown to be globulins it does not appear reasonable to assume that antipenicillinase is any exception. Furthermore, there is no reason to believe that the proteins of the antipenicillinase immune serum should react differently with penicillin than the proteins of normal serum. The second explanation for the ability of the antipenicillinase to inactivate penicillinase is a simple antigen-antibody combination between the penicillinase and the antipenicillinase. This, in turn, would allow the penicillin to work unhampered. This theory is supported by the specificity of the reactions in both the precipitation tests and the inhibition tests

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* Housewright, R.D. and Henry, R.J. Studied on Penicillinase. III. The effect of antipenicillinase on penicillin-resistant organisms. J. Bact., 1947, 53, 244.

reported here since the antipenicillinase immune serum of B. cereus only reacted with the penicillinases of B. cereus and B. megatherium, a closely related organism, and did not react with the penicillinases of Aërob. aërogenes and the acid-fast bacillus. Further support for this antibody-antigen theory is given by Housewright and Henry[22] who carried our manometric assays for penicillinase after a period of one hour's incubation with antipenicillinase. This assay depends upon the formation of penicilloic acid from penicillin, using penicillinase as a catalyst, and resulting in increased acidity of the penicillin molecule. By this method it was demonstrated that penicillinase incubated with antipenicillinase lost approximately 90 per cent of its activity.

One of the original reasons for undertaking this problem was to determine the practicability of antipenicillinase for clinical use. If the activity of penicillin against organisms which owed their resistance in whole or in part to the production of penicillinase, could be enhanced by the use of antipenicillinase the immune serum might be of value. The usefulness of this combination would depend upon the non-specificity of the antiserum in regard to the penicillinases produced by these organisms. Since the present work indicates the highly specific nature of antipenicillinase it is evident that this has little practical value since antipenicillinase would have to be produced for the penicillinase of each individual organism.

SUMMARY

1. A method is described for the production and isolation of penicillinase.

2. Antipenicillinase immune serum was produced in rabbits using *B. cereus* Penicillinase Schenley as the antigen.

3. Precipitin and inhibition tests were done using *B. cereus* Antipenicillinase immune serum and the penicillinases produced by *B. cereus* (Schenley), *B. cereus*, *B. megatherium*, a nonpathogenic acid-fast bacillus, and *Aërob. aërogenes*.

4. The precipitin reactions were positive for high dilutions of the *B. cereus* antipenicillinase immune serum and the penicillinases of *B. cereus* (Schenley), *B. cereus*, and *B. megatherium*. Similar tests were negative when the penicillinases of the acid-fast bacillus and *Aërob. aërogenes* were used.

5. The *B. cereus* antipenicillinase immune serum inactivated the penicillinases of *B. cereus* (Schenley), *B. cereus*, and *B. megatherium* and did not react with the penicillinases of the acid-fast bacillus and *Aërob. aërogenes*.

6. The results of the precipitin and inhibition tests indicate that *B. cereus* antipenicillinase immune serum has a high degree of specificity in its reactions with the penicillinase of *B. cereus* and the penicillinase of the closely related organism, *B. megatherium*. No such specificity was observed for its reaction with the penicillinases of the unrelated organisms, the acid-fast bacillus and *Aërob. aërogenes*.

TABLE I

Cup Assay for the Standardization of Penicillin

Assay read 1-21-48

	Units of Penicillin Vial A	25	5	2.5	1.5	1	.5
Plate 1	Zone of Inhibition in mm.	27	20	16	13	11	10
Plate 2	Zone of Inhibition in mm.	27	20	16	13	11	10

Assay read 1-22-48

	Units of Penicillin Vial B	25	5	2.5	1.5	1	.5
Plate 1	Zone of Inhibition in mm	27	20	16	13	11	10
Plate 2	Zone of Inhibition in mm	27	21	16	13	11	10

See Fig. 1 for photographs of sample cup assays, p. 48.

See Fig. 2 for standard curve for penicillin, p. 49.

TABLE II

Qualitative Assays for the Presence of Penicillinase

"Crude Penicillinase"	Zone of Inhibition in mm.
B. subtilis	11
B. subtilis (Marburg)	16.5
S. aureus	17
B. anthracis	13.5
Shig. dysenteriae (Shiga)	15.5
Shig. paradysenteriae (Flexner)	17
B. cereus	—
Acid-fast bacillus	—
B. megatherium	—
Aërob. aërogenes	—
Paracolon intermediate	—
E. coli communior	—
E. coli communis	—
Control	17

TABLE III

Immunization Schedule for Rabbits, Numbers 1-4

Rabbit	1	2	3	4
Type of Rabbit	Albino	Albino	Albino	Albino
Sex	Male	Male	Male	Male
8-26 Wt. in gms.	4500	4660	3730	5240
8-26 Penicillinase in units*	360	360	720	720
8-27	Dead	Dead	Dead	Rested
8-29				Dead

*Made up in sterile distilled water; 180 U per ml.

TABLE IV

Immunization Schedule for Rabbits, Numbers 5-8

Rabbit	5	6	7	8
Type of Rabbit	Albino	Albino	Gray Belgium Hare	Gray Belgium Hare
Sex	Male	Male	Male	Male
8-27 Wt. in gms.	4920	4590		
8-27 Penicillinase in units	100*			
8-29	100	100*		
8-30	100	100		
8-31	100	100		
9-1	100	100		
9-2	150	150		
9-3 Wt. in gms.			3680	3120
9-3	Anaphylactic shock***	150	50**	50**
9-4	Rested	150	50 in A.M. 50 in P.M.	50 in A.M. 50 in P.M.
9-5	50	150	50 50	50 50
9-6	50	150	50 75	50 75
9-7	50	150	50 75	50 75
9-8	100	175	75 75	75 75
9-9	100	175	75 75	75 75
9-10	125	200	100 75	100 75
9-11	125	200	100 75	100 75
9-12	150	200	100 100	100 100

* All penicillinase for Rabbits 5 and 6 was made up in sterile saline; 50 U per ml.

** All penicillinase for Rabbits 7 and 8 was made up in sterile distilled water; 50 U per ml.

*** Treated by subcutaneous injection of adrenalin.

TABLE IV (cont.)

Rabbit	5	6	7	8
9-13	150	200	100 100	100 100
9-14	150	200	100 100	100 100
9-15	175	200	100 100	100 100
9-16	175	200	100 100	100 100
9-17	175	200	100 100	100 100
9-18	175	200	100 100	100 100
Total units to date	2400 in 4 weeks	3450 in 4 weeks	2550 in 3 weeks	2550 in 3 weeks
9-19 Wt. in gms.	4470	3600	-	-
9-19	200	Rested	125 100	125 100
9-20	200	-	100 Sick	125 100
9-21	200	-	125 100	125 100
9-22	200	-	125 100	125 100
9-23	200	-	125 100	125 100
9-24	200	-	125 100	125 100
9-25	200	1st trial bleeding	125 100	125 100
9-26	200	Rested	125 100	125 100
9-27	200	-	125 100	125 100
9-28	200	-	125 100	125 100
9-29	200	-	125 100	125 100
9-30	200	-	125 100	125 100
10-1	200	-	125 100	125 100
10-2	200	-	125 100	125 100
10-3	200	-	125 100	125 100
10-4	200	-	125 100	125 100

TABLE IV (cont.)

Rabbit	5	6	7	8
10-5	200	-	125 100	125 100
10-6	200	-	125 100	125 100
10-7	200	-	125 100	125 100
10-8	200	-	125 100	125 100
10-9	200	-	125 100	125 100
10-10	200	-	125 100	125 100
10-11	200	-	125 100	125 100
10-12	200	-	125 100	125 100
10-13	200	-	125 100	125 100
10-14	200	2nd trial bleeding	125 100	125 100
10-15	200	Rested	125 100	125 100
10-16	200	-	125 100	125 100
10-17	200	-	125 100	125 100
10-18	200	-	125 100	Rested
10-19	200	-	Rested	-
10-20	200	-	-	-
10-21	200	-	-	-
10-22	200	-	-	-
10-23	Rested	-	-	-
Total units to date	9200	3450	9175	9075
10-27 Wt. in gms.	4500	4100	3500	3220
11-2	Rested	Rested	1st trial bleeding	1st trial bleeding
11-8	1st trial bleeding	-	-	-
11-20	-	3rd trial bleeding	-	-

TABLE IV (cont.)

Rabbit	5	6	7	8
12-8 Wt. in gms.	4400	3880	3210	3100
12-8	Total rest 6 wks. 4 days	Total rest 10 wks. 4 days	Total rest 7 wks. 1 day	Total rest 7 wks. 2 days
Booster dose				
12-9	25 25	25 25	25 25	25 25
12-10	50	50	50	50
12-11	100	100	100	100
12-12	150	150	150	150
12-13	200	200	200	200
12-14	200	200	200	200
12-15	Rested	Rested	Rested	Rested
12-22	2nd trial bleeding	4th trial bleeding	2nd trial bleeding	2nd trial bleeding
12-27	-	-	Dead	-
12-29 Wt. in gms.	4000	3800	2580	2820
Total loss of wt. in gms.	920	790	1100	300
12-29	Bled by cardiac puncture	Bled by cardiac puncture	-	Bled by cardiac puncture
Total units of penicillinase	9950	4200	9925	9825

TABLE V

A. Precipitin Tests using Antipenicillinase Immune Serum #6 and Penicillinase of B. cereus (Schenley)

Antipenicillinase Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	−
1:2	+	+	+	+	+	+	−
1:4	+	+	+	+	+	±	−
1:8	+	+	+	+	+	±	−
1:16	+	+	+	±	−	−	−
1:32	+	+	±	−	−	−	−
1:64	±	±	±	−	−	−	−
Control	−	−	−	−	−	−	−

B. Precipitin Tests using Normal Serum and Penicillinase B. cereus (Schenley)

Normal Serum Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	−
1:2	+	+	−	−	−	−	−
1:4	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	−
1:16	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

TABLE VI

A. Precipitin Tests using Antipenicillinase Immune Serum #6 and Penicillinase of *B. cereus*

Antipenicillinase Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	-
1:2	+	+	+	+	+	+	-
1:4	+	+	+	+	+	+	-
1:8	+	+	+	+	+	+	-
1:16	+	+	+	+	-	-	-
1:32	+	+	±	-	-	-	-
1:64	±	±	-	-	-	-	-
Control	-	-	-	-	-	-	-

B. Precipitin Tests using Normal Serum and Penicillinase of *B. cereus*

Normal Serum Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	-
1:2	+	-	-	-	-	-	-
1:4	-	-	-	-	-	-	-
1:8	-	-	-	-	-	-	-
1:16	-	-	-	-	-	-	-
1:32	-	-	-	-	-	-	-
1:64	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-

TABLE VII

A. Precipitin Tests using Antipenicillinase Immune Serum #6 and Penicillinase of B. megatherium

Antipenicillinase Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	−
1:2	+	+	+	+	+	+	−
1:4	+	+	+	+	+	±	−
1:8	+	+	+	+	±	±	−
1:16	+	+	±	±	±	±	−
1:32	±	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

B. Precipitin Tests using Normal Serum and Penicillinase of B. megatherium

Normal Serum Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	−
1:2	−	−	−	−	−	−	−
1:4	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	−
1:16	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

TABLE VIII

A. Precipitin Tests using Antipenicillinase Immune Serum #6 and Penicillinase of the Acid-fast Bacillus

Antipenicillinase Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	+	−
1:2	+	+	±	±	±	−	−
1:4	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	−
1:16	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

B. Precipitin Tests using Normal Serum and Penicillinase of the Acid-fast Bacillus

Normal Serum Dilution	Penicillinase Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	±	±	−
1:2	−	−	−	−	−	−	−
1:4	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	−
1:16	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

TABLE IX

A. Precipitin Tests using Antipenicillinase Immune Serum #6 and Penicillinase of Aërob. aërogenes

Antipenicillinase Dilution	Penicillinase Dilution 1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	+	+	+	±	−
1:2	+	−	−	−	−	−	−
1:4	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	−
1:16	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

B. Precipitin Tests using Normal Serum and Penicillinase of Aërob. aërogenes

Normal Serum Dilution	Penicillinase Dilution 1:2	1:4	1:8	1:16	1:32	1:64	Control
Straight	+	+	−	−	−	−	−
1:2	−	−	−	−	−	−	−
1:4	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	−
1:16	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−
1:64	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−

TABLE X

Inhibition Tests using the Penicillinase of Bacillus cereus (Schenley)

Dilution of Penicillinase	1:10	1:20	1:40	1:80	1:100	Control
Immune Serum #5	18*	22	24	25	27	27
Normal Serum	—	—	—	17	19	27
No Serum	—	—	13	20	22	27
Immune Serum #6	23	27	27	27	27	27
Normal Serum	—	—	—	17	19	27
No Serum	—	—	13	20	22	27
Immune Serum #7	25	25	26	27	27	27
Normal Serum	—	—	—	17	19	27
No Serum	—	—	13	20	22	27
Immune Serum #8	—	17	24	27	27	27
Normal Serum	—	—	—	17	19	27
No Serum	—	—	13	20	22	27

*The diameter of the zone of inhibition is given in mm.

TABLE XI

Inhibition Tests using the Penicillinase of *Bacillus cereus*

Dilution of Penicillinase	1:10	1:20	1:40	1;80	1:100	Control
Immune Serum #5	18*	23.5	25	26	27	27
Normal Serum	—	—	—	15.5	17	27
No Serum	—	—	—	17	20	27
Immune Serum #6	24	26.5	26.5	27	27	27
Normal Serum	—	—	—	15.5	17	27
No Serum	—	—	—	17	20	27
Immune Serum #8	—	18	23	26	27	27
Normal Serum	—	—	—	15.5	17	27
No Serum	—	—	—	17	20	27

*The diameter of the zone of inhibition is given in mm.

See Fig. 3 for photographs of sample cup assays, p. 50.

TABLE XII

Inhibition Tests using the Penicillinase of *Bacillus megatherium*

Dilution of Penicillinase	1:10	1:20	1:40	1:80	1:100	Control
Immune Serum #5	25*	26.5	27	27	27	27
Normal Serum	14	19	23	24	26.5	27
No Serum	17	21	24	25	27	27
Immune Serum #6	26	27	27	27	27	27
Normal Serum	14	19	23	24	26.5	27
No Serum	17	21	24	25	27	27
Immune Serum #8	25.5	26.5	27	27	27	27
Normal Serum	14	19	23	24	26.5	27
No Serum	17	21	24	25	27	27

*The diameter of the zone of inhibition is given in mm.

See Fig. 4 for photographs of sample cup assays, p. 51.

TABLE XIII

Inhibition Tests using the Penicillinase of the Acid-fast Bacillus

Dilution of Penicillinase	1:10	1:20	1:40	1:80	1:100	Control
Immune Serum #5	—	16*	23	26	27	27
Normal Serum	—	16	23.5	26	27	27
No Serum	—	16	23	26	27	27
Immune Serum #6	—	16	23	26	27	27
Normal Serum	—	16	23.5	26	27	27
No Serum	—	16	23	26	27	27
Immune Serum #8	—	16	22.5	25.5	27	27
Normal Serum	—	16	23.5	26	27	27
No Serum	—	16	23	26	27	27

*The diameter of the zone of inhibition is given in mm.

See Fig. 5 for photographs of sample cup assay, p. 52.

TABLE XIV

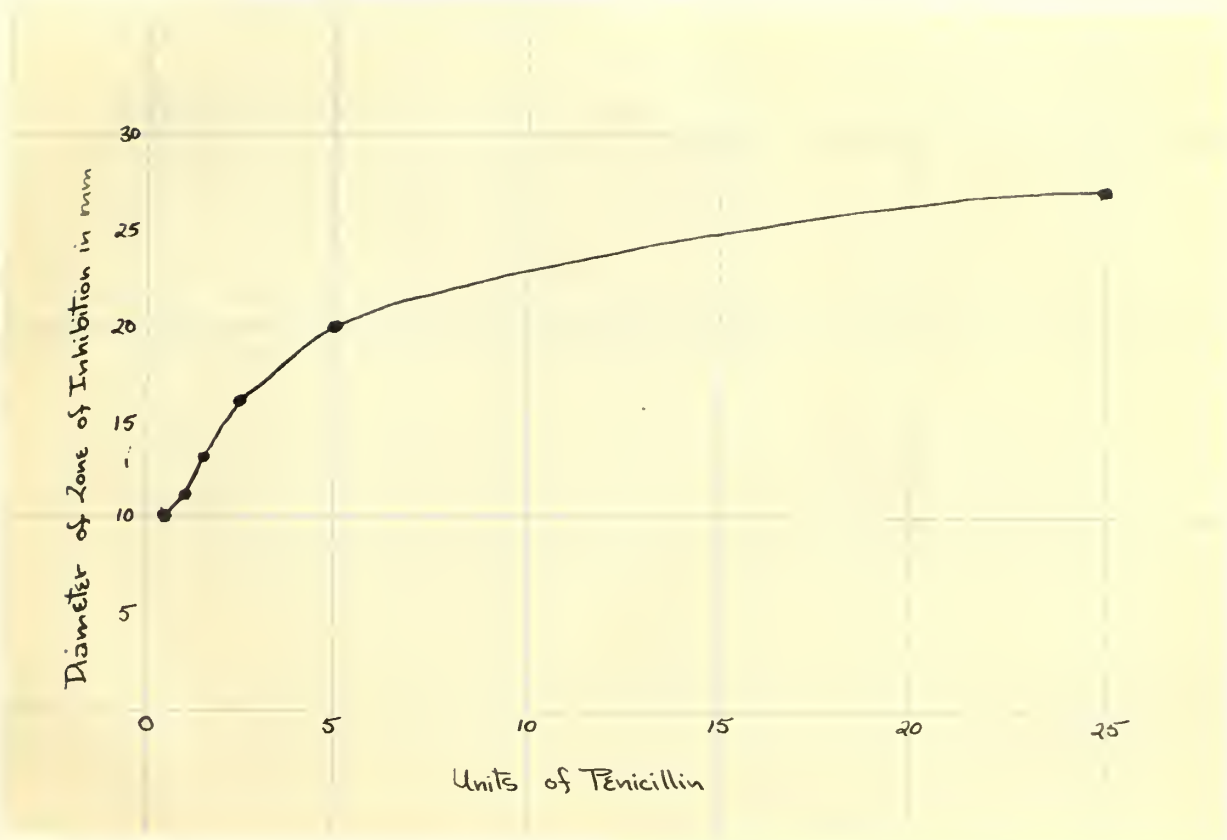
Inhibition Tests using the Penicillinase of "Aerobacter aerogenes"

Dilution of Penicillinase	1:10	1:20	1:40	1:80	1:100	Control
Immune Serum #5	—	—	14*	20.5	24	27
Normal Serum	—	—	14	21	24	27
No Serum	—	—	14	21	24	27
Immune Serum #6	—	—	13	19	23.5	27
Normal Serum	—	—	14	21	24	27
No Serum	—	—	14	21	24	27
Immune Serum #8	—	—	14	20.5	24	27
Normal Serum	—	—	14	21	24	27
No Serum	—	—	14	21	24	27

*The diameter of the zone of inhibition is given in mm.

Fig. 1. Cup Assay for Penicillin. Plate shows zones of inhibition for the standard curve after 20 hours inhibition at 30 degrees C. See Table I, p. 31 and Fig. 2, p. 49.

Fig. 2. Standard Curve for Penicillin



See Table 1, p. 31.

Fig. 3. Cup Assay of the Inhibition Tests using the Penicillinase of B.cereus

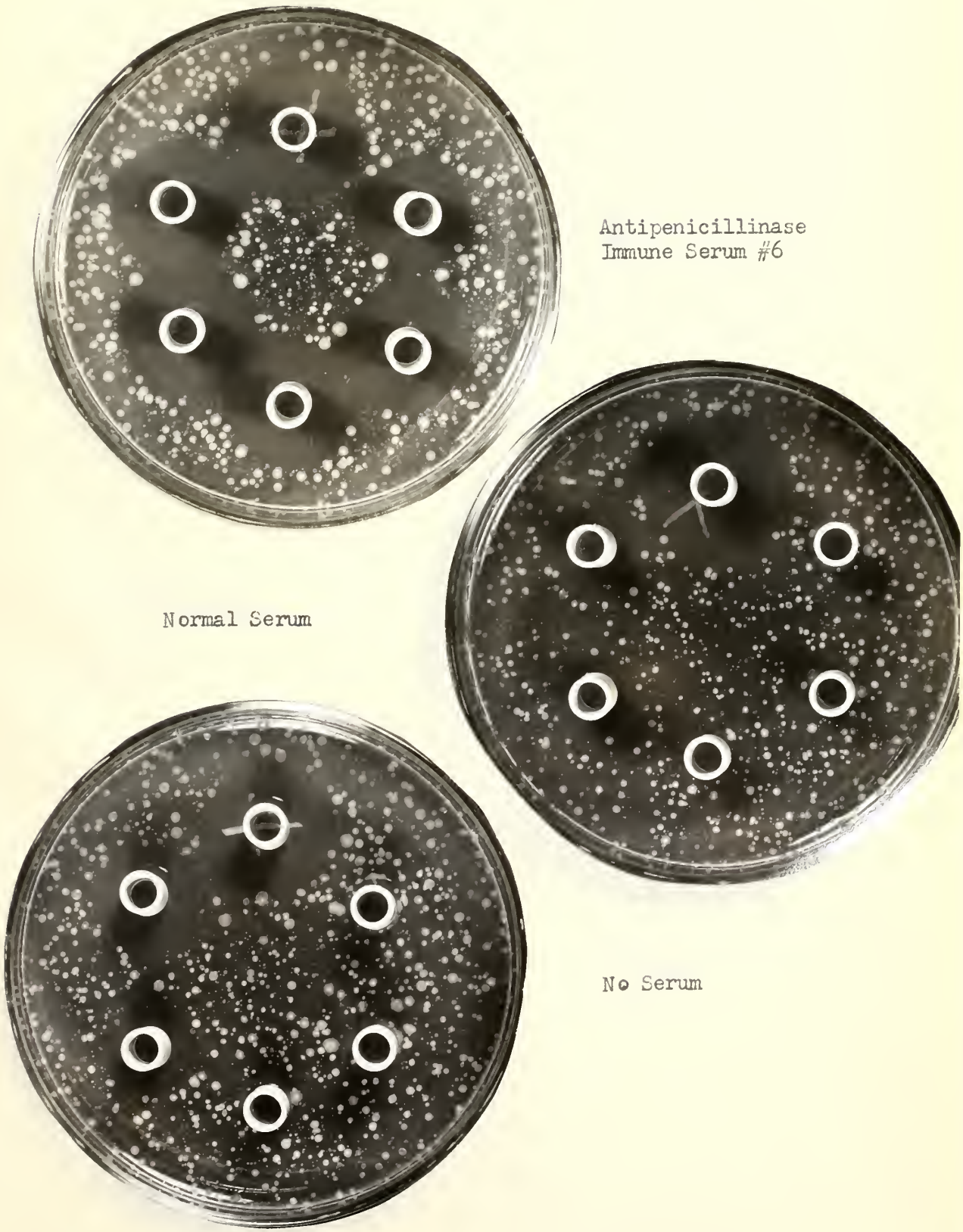


Fig. 4. Cup Assay of the Inhibition Tests using the Penicillinase of B.megatherium

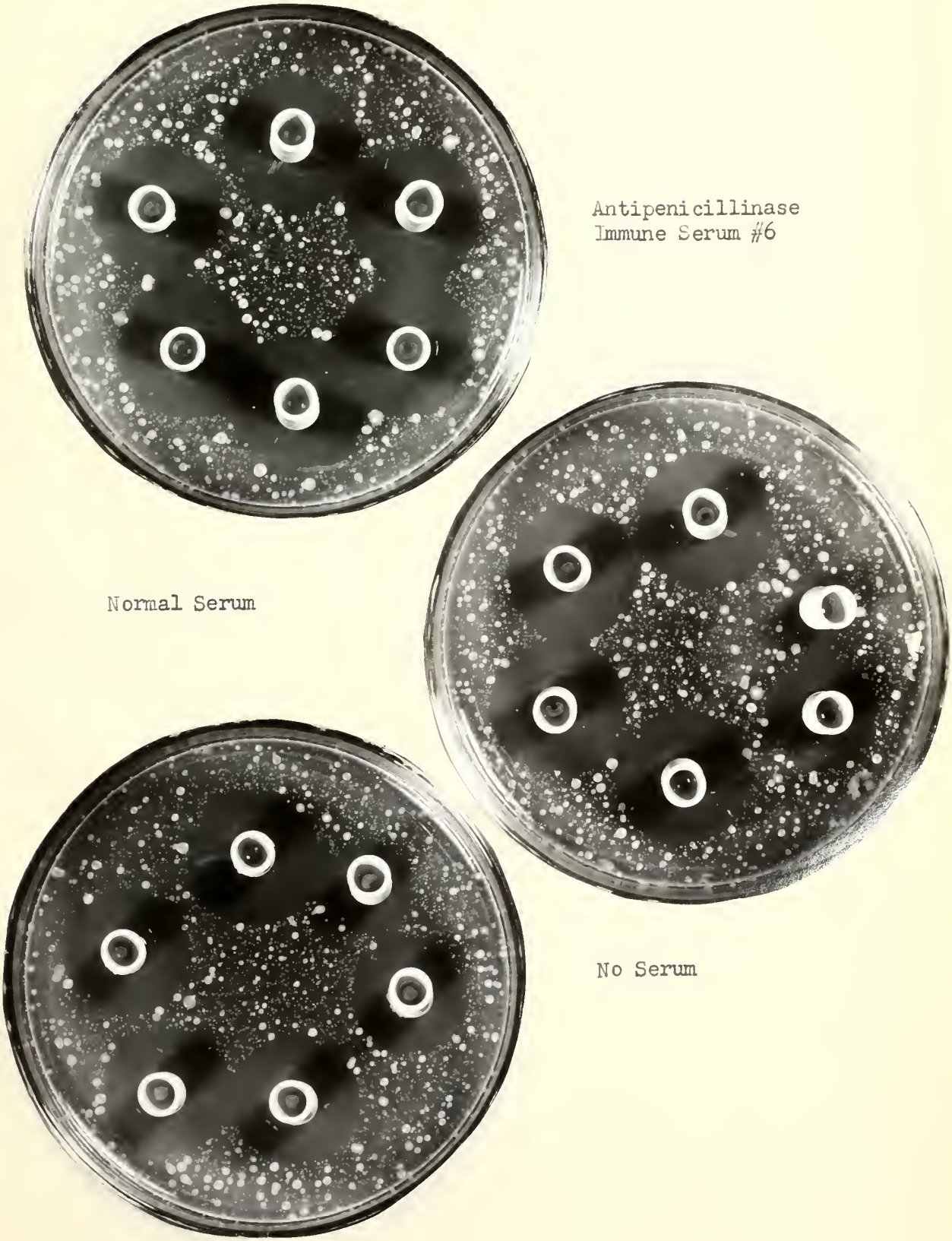
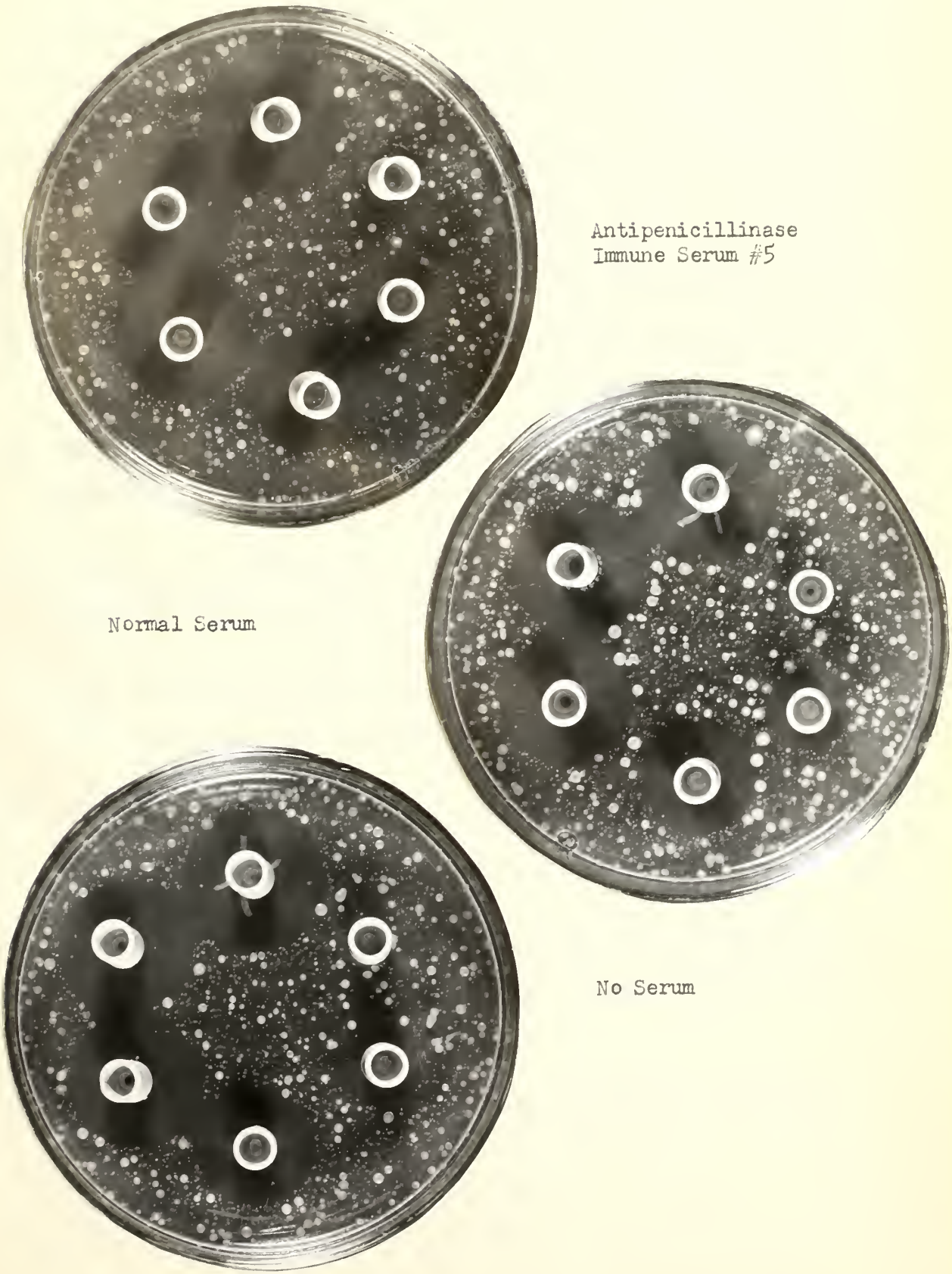


Fig. 5. Cup Assay of the Inhibition Tests using the Penicillinase of the Acid-fast Bacillus



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COMPREHENSIVE ABSTRACT OF THE DISSERTATION

The purpose of the present problem was to determine the specificity of antipenicillinase immune serum. It has been shown that some organisms produce an enzyme, penicillinase, which is capable of inactivating penicillin. Perlstein and Liebmann (1945) and later Housewright and Henry (1947) produced an antipenicillinase immune serum by the repeated intravenous injection of penicillinase into rabbits and measured the antibody response by precipitin and inhibition tests. This antipenicillinase immune serum was able to protect penicillin from destruction by the same penicillinase which was used as an antigen for the production of the immune serum. Normal rabbit serum did not possess this property.

Housewright and Henry stated that antipenicillinase immune serum produced by the injection of penicillinase elaborated by B. cereus B-569, inactivated penicillinase produced by B. cereus B-569 and by S. aureus Long III A, and from this concluded that penicillinases from these two sources were immunologically similar.

This is the report of the production of antipenicillinase immune serum using penicillinase produced by one organism and the determination of its specificity for penicillinases produced by three other organisms. This specificity or nonspecificity was determined by precipitin and inhibition tests.

Four organisms, B. cereus, B. megatherium, a rapidly growing, nonpathogenic, acid-fast bacillus, and Aërobacter aërogenes were found

to produce penicillinase in sufficient quantities for the purpose of the experiment. These organisms were grown in casamino acid medium at pH 7. Penicillin sodium (Bristol) was added in the proportion of 200 units per ml of medium 20 minutes after the medium was inoculated and an identical amount 24 hours later. The cultures were incubated at 37 C for 120 hours and were agitated at frequent intervals. They were then centrifugalized at 4,500 rpm at 50 F for 1 hour in order to remove the organisms. Then the crude penicillinase was decanted. The penicillinase was precipitated as a finely diffuse precipitate by reducing the pH to 5.2 with glacial acetic acid. The material was then put through fritted glass filters of F porosity and the filtrate discarded. The precipitated penicillinase remaining on the surface of the filter was redissolved in 0.5 per cent ammonia water, the quantity used being such as to concentrate the penicillinase 10 times. The pH was then adjusted to 7 with glacial acetic acid and the purified penicillinase was stored at 5 C for assay.

Penicillinase produced by B. cereus NRRL B-569* was used as an antigen for the preparation of antipenicillinase immune serum. Three rabbits were given approximately 10,000 units each and one rabbit received 4200 units. It was later found that there was no appreciable difference in the titer of the serum of the animal receiving the smaller amount and those receiving the larger. Trial assays were run at intervals. The rabbits were rested 2 weeks and then bled from the heart. The rabbits

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* The penicillinase used was obtained from the Schenley Laboratories

were fasted for 15 hours before bleeding. The blood was left at room temperature for 6 hours to allow the clots to retract and was placed in the ice box overnight. The following morning the bloods were centrifugalized and the sera decanted and allowed to "age" one week.

The penicillinase was assayed quantitatively using a modification of the Oxford cup method. A further modification of this same method was used for the inhibition tests with antipenicillinase. The inhibition tests were performed using a standard concentration of antipenicillinase immune serum and diluting the penicillinase. Precipitin tests using antipenicillinase immune serum and the various penicillinases were done using a capillary-tube "ring" method.

The antipenicillinase immune serum produced against B. cereus Penicillinase Schenley inactivated the penicillinases produced by B. cereus (Schenley), B. cereus, and B. megatherium. There was no evidence that the antipenicillinase immune serum of B. cereus inhibited the penicillinase produced by the acid-fast bacillus and Aërob. aërogenes. These findings were confirmed by the precipitin tests in which the antipenicillinase immune serum of B. cereus reacted strongly with B. cereus (Schenley), B. cereus, and B. megatherium penicillinases while no significant reaction was observed with the penicillinases of the acid-fast bacillus and Aërob. aërogenes. It was concluded that penicillinases of B. cereus and B. megatherium were immunologically similar and the penicillinases of the acid-fast bacillus and Aërob. aërogenes were immunologically dissimilar to the penicillinase of B. cereus.

It is not surprising to find that the penicillinases of *B. cereus* and *B. megatherium* are immunologically similar since these two organisms are closely related both belonging to the family Bacillaceae and the genus *Bacillus*. Further, the organisms are so closely related that intermediate forms between the two have been described. The other two organisms tested have little in common with *B. cereus*.

One of the original reasons for undertaking this problem was to determine the practicability of antipenicillinase for clinical use. If the activity of penicillin against organisms which owed their resistance in whole or in part to the production of penicillinase could be enhanced by the use of antipenicillinase, the immune serum might be of value. The usefulness of this combination would depend upon the non-specificity of the antiserum in regard to the penicillinase produced by these organisms. Since the present work indicates the highly specific nature of antipenicillinase it is evident that this has little practical value since antipenicillinase would have to be produced for the penicillinase of each individual organism.

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